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Altered (Copy-Up) Forms of Initiator Protein π Suppress the Point Mutations Inactivating the γ Origin of Plasmid R6K

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The R6K γ origin core contains the P2 promoter, whose −10 and −35 hexamers overlap two of the seven binding sites for the R6K-encoded π protein. Two mutations, P2-201 and P2-203, which lie within the −35 region of P2, are shown to confer a promoter-down phenotype. We demonstrate here that these mutations prevent replication of a γ origin core plasmid. To determine whether or not the reduced promoter activity caused by these mutations is responsible for their effect on replication, we generated two new mutations (P2-245-6-7 and P2-246) in the −10 hexamer of the P2 promoter. Although these new mutations inhibit P2 activity as much as the P2-201 and P2-203 mutations, they do not prevent replication of the γ origin core. Therefore, activity of the P2 promoter does not appear to be required for replication. We also show that the inability of the γ origin to function in the presence of the P2-201 and P2-203 mutations is reversed by the hyperactive variants of π protein called copy-up π. This suppression occurs despite the fact that in vivo dimethyl sulfate methylation protection patterns of the γ origin iterons are identical in cells producing wild-type π and those producing copy-up π variants. We discuss how the P2-201 and P2-203 mutations could inhibit replication of the γ origin core and what mechanisms might allow the copy-up π mutants to suppress this deficiency.

Escherichia coli plasmid R6K carries a 5.5-kb replication region which contains three origins of replication: α, β, and γ (5, 24, 28, 31). All three origins require initiator protein π, encoded by the *pir* gene of R6K (24, 28, 38, 45, 46). When the flanking α and β sequences are removed, the remaining γ origin can replicate autonomously (18, 48, 51) if the *pir* gene is provided either in *cis* or in *trans* (23, 28). The α and β origins require a 277-bp fragment of the γ origin in *cis* for their activity (45, 46, 49). The γ origin contains nucleotide sequence repeats (iterons) to which π binds in vivo and in vitro (15, 20, 21, 36). Binding sites for the integration host factor IHF (9a) and DnaA (52) proteins and two promoter sequences, P1 and P2 (37, 50), are also embedded within the γ origin (Fig. 1A).

Initiator protein π acts as an activator of replication when present at a low level (23, 28) and as an inhibitor of replication when its intracellular concentration increases (6, 13, 14). Certain hyperactive ("copy-up") mutants of π can activate the γ origin more efficiently than the equivalent amount of wild-type (wt) π. The mechanism responsible for the elevated initiator activity of these altered proteins is not known.

The γ origin responds to the intracellular concentration of π protein and contains elements allowing its maintenance at a defined plasmid copy number (14, 27; for a recent review, see reference 11). Work done in our laboratory has demonstrated that this origin can be separated into two segments, as shown in Fig. 1A: (i) a 106-bp DNA segment located to the left of nucleotide (nt) +1 of the γ origin which can be deleted if the intracellular level of π protein is decreased (52) and (ii) the remaining 277-bp fragment containing all of the *cis*-acting elements necessary for replication. We have coined the terms "γ origin core" for the 277-bp DNA segment and "enhancer" for the 106-bp DNA segment to the left of the core. We have used the core segment to study the mechanism regulating the initiation frequency of an iteron-containing molecule (11).

High levels of wt π inhibit γ origin core replication (52) and also repress the activity of the P2 promoter (37). Thus, we considered whether π protein might inhibit replication by repressing transcription originating within the γ origin core. To test this model, we carried out a detailed analysis of the effects of two previously isolated mutations, P2-201 and P2-203. Both mutations are promoter-down mutations that map in the −35 region of the P2 promoter (Fig. 1B) (37). We tested the activity of the P2 promoter and the replication properties of the γ origin plasmids containing these mutations. Moreover, we generated two new mutations which decrease the number of matches to the consensus at the −10 hexamer of the P2 promoter. Although these new mutations also confer a P2 promoter-down phenotype, they do not interfere with the replication of the γ origin core. Thus, the P2-201 and P2-203 mutations are unlikely to prevent replication of the γ origin core by interfering with transcription. We showed that the function of the γ origin core containing either the P2-201 or the P2-203 mutation can be restored by providing copy-up variants of π instead of wt π. By using the in vivo methylation protection assay, we tested whether wt and copy-up π differ in their interactions with the iterons. We found that they do not. Thus, we concluded that wt π and copy-up mutant π differ in the basic requirements for the activity of the γ origin core, although this difference is not directly related to their binding activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in these studies are listed in Table 1.

Transformations. Competent cells were transformed with supercoiled plasmid DNA by using the calcium chloride procedure (42).

Determination of plasmid copy number. Plasmid DNA was isolated by the alkaline lysis procedure as previously described (33). DNA was linearized with *Sna*BI and run on a 1% agarose gel in TAE buffer (50 mM Tris, 10 mM

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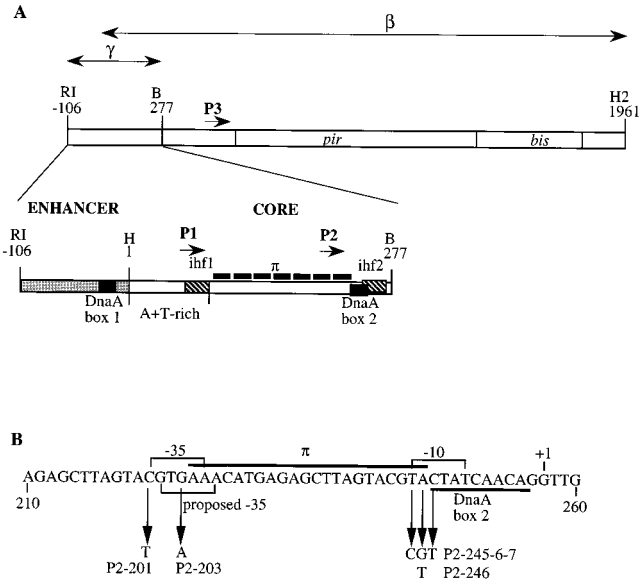

FIG. 1. (A) The replication region of plasmid R6K encompassing the γ and β origins. *cis*-acting sequences required for replication of the γ and β origins are indicated by double-headed arrows. The positions of the π-encoding *pir* gene and its promoter (P3) and the *bis* gene are shown. Shown in the middle is the γ origin, consisting of a 106-bp enhancer and a 277-bp core. The 277-bp core segment, required for γ and β origins, contains an A+T-rich region and seven 22-bp direct repeats (the iterons), indicated by black bars, to which π binds (13, 15, 19, 36). In addition, two IHF-binding sites, ihf1 and ihf2, are represented by hatched boxes (9a, 12), DnaA-binding site box 2 is shown as a solid box (32, 52), and the P1 and P2 promoters are indicated by two arrows (37, 50). The 106-bp enhancer contains DnaA-binding site box 1 (52). B, H, H2, and RI represent recognition sites for restriction enzymes *Bgl*II, *Hin*dIII, *Hae*II, and *Eco*RI, respectively. (B) The γ origin sequence from positions +210 to +260. The positions of −35 and −10 hexamers of the P2 promoter are indicated by brackets, and the thick line indicates π binding site 7 and DnaA box 2. The 5′ end of the P2 transcript, as defined by these studies, is designated +1. The positions and base substitutions of the P2-201, P2-203 (37), P2-246, and P2-245-6-7 mutations are shown. The reassigned −35 hexamer of P2 is indicated by the lower bracket labeled "proposed −35."

Na_2-EDTA, 30 mM Na-acetate, pH adjusted to 8.2 with glacial acetic acid). Negatives of the gel photographs (Polaroid film type 55) were scanned with a Hoefer Scientific Instruments scanning densitometer GS300 using the program GS370 data system (Apple MacIntosh Version).

DNase I protection assay. Plasmids pMF32 and pMU4 were digested with *Eco*RI or *Hin*dIII. The 3′ ends were labeled with Sequenase (U.S. Biochemical Corp.) in the presence of [^{32}P]ATP. The *Eco*RI-labeled fragments were then digested with *Hin*dIII; the *Hin*dIII-labeled fragments were digested with *Eco*RI. The conditions for DNase I protection and DNA sequencing experiments were as described earlier (15).

DNA sequencing. Sequencing of double-stranded DNA was performed as described by Sanger et al. (43), with a Sequenase II kit (U.S. Biochemical Corp.).

RNA isolation and primer extension. Plasmid-encoded RNAs were prepared as described by Karls et al. (26). RNA primer extension reactions were carried out as described by Inoue and Cech (22) by using a 5′-end-labeled −40 universal primer (U.S. Biochemical Corp.), except that the hybridization step was done at 45°C for 15 min and primer extension was done at 45°C in the presence of actinomycin D (100 μg/ml). To compare the relative amounts of the P2 transcripts under different conditions, we used plasmid pMSB10 encoding a reference transcript as an internal control. This plasmid contains a strong ribosomal *rrnB* P1 promoter and a sequence complementary to the −40 universal sequencing primer. Two sets of cultures, one containing cells with control plasmid pMSB10 and the other carrying pMU9 or pMU10, were grown to the log phase. At that point, the two cultures were mixed at a ratio of 1:20 (50 μl of the culture containing cells with pMSB10 and 1 ml of the culture containing cells with pMU9 or pMU10) and quickly chilled. Total RNA was isolated from the mixture by following the procedure described above. The −40 sequencing primer was used for reverse transcription of transcripts from P2 and the *rrnB* P1 reference promoter. The primer extension product from the *rrnB* P1 transcript is 40 nt longer than the primer extension product from the P2 transcript; thus, the two products can be separated on a sequencing gel. The amount of the pMSB10 transcript was expected to remain constant in all of the samples tested, while the amount of the P2 transcript would vary depending on the activity of the P2 promoter.

The transcript amount was quantified by measuring radioactivity on a Betascope 603 blot analyzer (Intelligenetics-Betagen), and the signals were normalized to the *rrnB* P1 control transcript.

Site-directed mutagenesis. The unique-site elimination mutagenesis technique (8) was employed to introduce changes into pMU9, which carries the R6K γ origin core cloned between two sets of transcriptional terminators in pMSB1. Oligonucleotides 5′-GCTTAGTACG**TTCTAT**CAACAGG and 5′-GAGCTTA GTACG**CGTTAT**CAACAGGTTG were used to alter the −10 hexamer of the P2 promoter (in boldface with mutant nucleotides underlined) and eliminate the *Sna*BI site in this region to select for mutants. This procedure resulted in plasmid pMU11 carrying the single mutation P2-246 (first oligonucleotide) and pMU12 carrying multiple mutations P2-245-6-7 (second oligonucleotide). To produce another −10 hexamer mutant which does not affect the partially overlapping DnaA box 2 sequence but still eliminates the *Sna*BI site (used to select for mutants), nt 2 contained three changes. DNA sequencing by the dideoxy method

TABLE 1. Plasmids and *E. coli* strains used in this study

Plasmid or strain	Description or genetic markers	Source or reference
Plasmids		
Δ14-*pir*wt, Δ14-*pir*113, Δ14-*pir*200	RK2 *ori*, Tet^r	14, 52
Δ22-*pir*wt, Δ22-*pir*113, Δ22-*pir*405-cos, Δ22-*pir*13, Δ22-*pir*1, Δ22-*pir*104, Δ22-*pir*116, Δ22-*pir*200	RK2 *ori*, Tet^r	14, 35, 52
pMF32	pUC9 containing 277-bp *Hin*dIII-*Bam*HI fragment of R6K γ *ori*, wt P2, Pen^r	52
pMU3	pMF32 carrying P2-203	This study
pMU4	pMF32 carrying P2-201	This study
pMF33	pUC8 carrying 383-bp *Eco*RI-*Bgl*II fragment of R6K γ *ori* containing wt P2	52
pMU8	pMF33 carrying P2-201	This study
pMSB1	pBR322 *ori* carrying two sets of T1 terminators; one set contains 4 terminators, the other contains 1	2
pMU9	pMSB1 carrying wt P2, Pen^r	This study
pMU10	pMSB1 carrying P2-201, Pen^r	This study
pMU11	pMSB1 carrying P2-246, Pen^r	This study
pMU12	pMSB1 carrying P2-245-6-7, Pen^r	This study
pMSB10	pMSB1 carrying *rrnB* P1 with −46 to +50 bp upstream sequence, Pen^r	2a
E. coli strains		
p3478	F$^-$ λ$^-$ *thyA36 deoC2* IN(*rrnD-rrnE*)*1 polA1*	7
W3110	F$^-$ λ$^-$ *thyA36 deoC2* IN(*rrnD-rrnE*)*1 polA*$^+$	7
TB1	F$^-$ *ara* Δ(*lac proAB*) *rpsL hsdR17*(r_K^- m_K^+) ϕ80d*lacZ*ΔM15	1
LE392	*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*	3, 39

TABLE 2. Transformation efficiency of plasmids with wt or mutant γ origin into *polA*$^+$ and *polA* mutant strains

Transforming plasmid DNA	γ *ori* composition	Transformation efficiency[a]	
		polA$^+$ W3110 (Δ14-*pir*wt)	*polA* mutant p3478(Δ14-*pir*wt)
pMF32	wt P2 core	1.9×10^3	5.1×10^2
pMU3	P2-203 core	1.7×10^3	0
pMU4	P2-201 core	2.1×10^3	0
pMF33	wt P2 core + enhancer	1.2×10^3	7.5×10^3
pMU8	P2-201 core + enhancer	NT[b]	5.9×10^3

[a] 10^8 competent cells were transformed with 200 ng of the indicated plasmid DNA and plated on Luria-Bertani medium plates containing tetracycline (15 μg/ml) and penicillin (250 μg/ml). Transformation efficiency is expressed in number of colonies per microgram of DNA per 10^7 cells plated in one experiment. Similar values were obtained in four other experiments.

[b] NT, not tested.

(see above) was used to verify the P2 promoter mutations and ensure that no other mutations were introduced.

In vivo DNA modification and primer extension analysis. In vivo DNA modification by dimethyl sulfate (DMS) and subsequent analysis by primer extension analysis were performed essentially as described previously (4, 16, 17, 44). Briefly, cells harboring pMF33, without or with each of the different plasmids expressing the various π proteins, were grown in Luria-Bertani medium supplemented with the appropriate antibiotics (optical density at 550 nm, about 0.6). The cells were then treated with 10 mM DMS for 5 min before DNA extraction. Primer extension analysis was done with a labeled universal M13 primer (New England Biolabs).

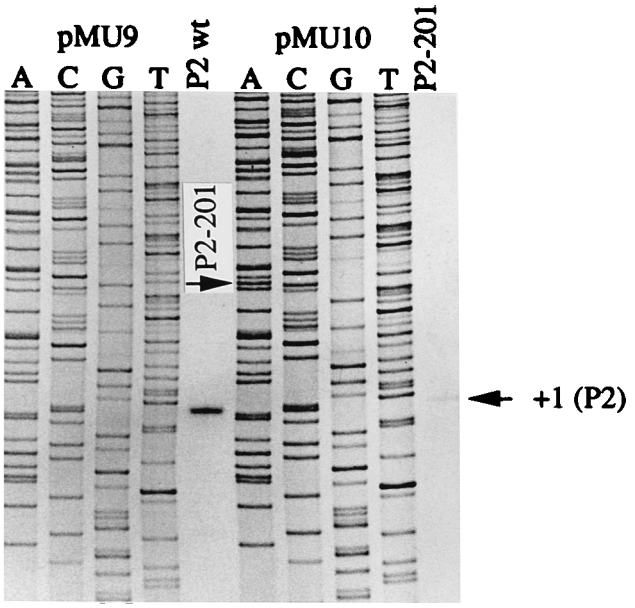

FIG. 2. Mapping of the 5′ end of the P2 RNA from plasmids pMU9 (lane P2 wt) and pMU10 (lane P2-201) by primer extension. Lanes A, C, G, and T represent the sequences of plasmids pMU9 and pMU10 (as indicated) determined with the −40 universal sequencing primer. The arrow labeled "+1 (P2)" points to the extended primer DNA indicating the 5′ end of the P2 transcript.

RESULTS

Mutations P2-201 and P2-203 prevent replication of the γ origin core plasmids. As stated above, the P2-201 and P2-203 mutations and high levels of π reduce P2 promoter activity. In addition, high levels of π repress γ origin core replication. Here we tested whether P2-201 and P2-203 also inhibit replication of γ origin core-containing plasmids. We tested this with a simple experimental system consisting of a series of isogenic plasmids. Plasmids pMU3 and pMU4 carry the previously characterized P2-203 and P2-201 mutations, respectively, while pMF32 has the wt P2 promoter sequence. Importantly, each of these plasmids, as well as several others listed in Table 1, are hybrid replicons, carrying a non-R6K origin (pUC) in addition to the γ origin. This feature allowed us to construct some clones with a nonfunctional γ origin core. We tested the function of the γ origin core by conditionally turning off the pUC origin, which requires DNA polymerase I, encoded by the *polA* gene. Transformations were performed with the following isogenic strains: W3110 *polA*$^+$(Δ14 *pir*) and p3478 *polA* mutant (Δ14 *pir*). The Δ14 helper plasmid produces a low level of wt π that allows replication of pMF32 in a *polA* mutant host (52). As shown in Table 2A, plasmids pMF32, pMU3, and pMU4 all transformed the *polA*$^+$ strain, as expected. In contrast, transformants of the *polA* mutant strain were obtained with pMF32 but not with pMU3 or pMU4. Thus, the P2-201 and P2-203 mutations prevent replication of the γ origin core.

Next, we tested whether the enhancer can reverse the effect of these two promoter-down mutations. The enhancer is known to compensate for many conditions which normally inhibit replication (6a, 52). We used plasmids pMF33 and pMU8, which carry the enhancer in addition to the core; pMF33 has a wt P2 promoter, while pMU8 contains one of the promoter-down mutations (P2-201). We found that both pMU8 and pMF33 replicated in the *polA* mutant host carrying the Δ14 helper plasmid. Thus, the enhancer can reverse the effect of the P2-201 mutation on replication of the γ origin.

Mapping and quantification of P2 promoter-driven transcripts. Gene fusion experiments containing various segments of the γ origin core have shown an approximate position of the P2 promoter (37). We carried out an additional analysis of the P2 promoter at the transcriptional level for three reasons. First, P2 is a very weak promoter and indirect measurements of its strength with *galK* fusions gave readings of galactokinase activity only slightly above the background (37). Second, we were uncertain if our assignment of the −10 and −35 hexamers was correct (see Discussion). Third, we intended to resolve conflicting reports about transcriptional activity arising from within the γ origin core (40, 41, 47, 50). Another group of investigators has claimed that left-to-right transcription starts within the γ origin core from a segment approximately a hundred nucleotides upstream of the P2 promoter (41).

To map the 5′ end of the P2 transcript by primer extension (see Materials and Methods), the wt γ origin core was cloned downstream of a set of four tandem transcriptional terminators in pMSB1 (2) to produce plasmid pMU9 (Table 1). These terminators prevent substantial transcriptional readthrough from the vector sequences. (Such readthrough transcription was detected in γ origin core plasmids lacking terminators, i.e., pMU3 and pMU4 [data not shown].)

Total cellular RNA isolated from TB1 cells containing pMU9 was annealed to a primer complementary to a site 140 bp downstream of the γ origin fragment and extended as described in Materials and Methods. The start of the cDNA fragment corresponds to nt +257 in a sequencing sample of the γ origin core (Fig. 2). Thus, the first transcribed nucleotide (+1) is 10 nt away from the center of the previously assigned −10 hexamer (37). Similar assays were carried out with plasmid pMU10 (Fig. 2), which is isogenic to pMU9 (Table 1) but contains the P2-201 mutation. It should be mentioned that the flanking transcriptional terminators do not alter the replication properties (Tables 2 and 3) of the wt or mutant γ origin core.

To quantify the effect of the P2 promoter-down mutations,

TABLE 3. Transformation efficiency of plasmids with wt or mutant γ origin core into *polA*$^+$ and *polA* mutant strains

Transforming plasmid DNA	γ *ori* composition	Transformation efficiency[a]	
		polA$^+$ W3110 (Δ14-*pir*wt)	*polA* mutant p3478(Δ14-*pir*wt)
pMU9	wt P2 core	3.1×10^3	7.7×10^3
pMU10	P2-203 core	4.8×10^2	0
pMU11	P2-246 core	3.1×10^2	1.4×10^2
pMU12	P2-245 core	5.7×10^2	1.1×10^3

[a] 10^8 competent cells were transformed with 200 ng of the indicated plasmid DNA and plated on Luria-Bertani medium plates containing tetracycline (15 μg/ml) and penicillin (250 μg/ml). Transformation efficiency is expressed as number of colonies per microgram of DNA per 10^7 cells plated in one experiment. Similar values were obtained in four other experiments.

we used a transcript from the *rrnB* P1 promoter (−46 to +50) on plasmid pMSB10 (Table 1 and 2) as an internal reference. This transcript is 40 bp longer than the P2 transcript; therefore, the same primer could be used to detect both *rrnB* P1 and γ origin P2 transcripts. This procedure (described in Materials and Methods) permitted assessment of experimental error originating from variability in RNA isolation and primer annealing. Total cellular RNA was isolated from a mixture of TB1 cells carrying plasmid pMSB10 and cells containing either pMU9 or pMU10 at the ratios indicated in Materials and Methods. The results from several gels (a representative autoradiogram is shown in Fig. 3) showed that the P2-201 mutation decreases P2 promoter activity four- to fivefold. Moreover, our calculations indicate that P2 is 200-fold weaker than the *rrnB* P1 promoter. The precise mapping of the 5′ end of the P2-driven transcript allowed us to make an informed guess regarding the positions of the −10 and −35 hexamers of the P2 promoter. It turned out that our previous assignment was incorrect and that one of the promoter-down mutations lies outside the −35 consensus (see Discussion). Moreover, it is also clear that any transcripts produced from the P1 promoter (Fig. 1) (37, 50), or any other site within the γ origin core, must either terminate within the γ origin core or be produced at undetectable levels.

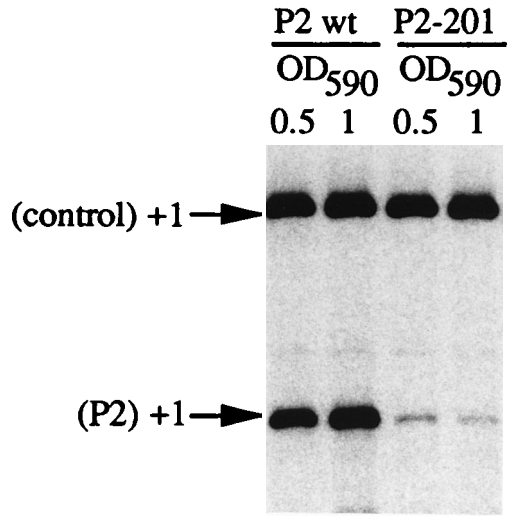

FIG. 3. Quantification of the P2 and P2-201 RNA transcripts by primer extension assay. Lanes represent primer extension reactions of RNA extracted from *polA*$^+$ cells carrying control plasmid pMSB10 or a γ origin core plasmid with the wt P2 promoter or the P2-201 mutant promoter. Samples were obtained at two different cell densities, as indicated (OD_{590}, optical density at 590 nm). Sample volumes were adjusted in accordance with the number of cells so that RNA in each sample was extracted from the same number of cells. Cells carrying control plasmid pMSB10 or an experimental γ origin core plasmid were mixed at a ratio of 1:20. The +1 (control) arrow indicates primer extension products from the *rrnB* P1 control transcript, and the +1 (P2) arrow points to the primer extension products from the wt P2 or P2-201 transcript.

Mutations in the −10 hexamer of the P2 promoter do not affect replication. The results presented above suggested that P2 promoter activity might be required for γ origin core replication and that elevated levels of π which inhibit γ origin core replication may do so by repressing the activity of the P2 promoter. If this hypothesis is correct, then one would expect promoter-down mutations within the −10 hexamer also to render the γ origin core inactive. We introduced such mutations at the most conserved nucleotides of the −10 hexamer of the P2 promoter, creating core plasmids pMU11 and pMU12, which carry mutations at positions 246 and 245-6-7, respectively (Table 1 and Fig. 1B). Plasmids pMU11 and pMU12 were then used in a transformation test. Surprisingly, both plasmids can transform either the *polA*$^+$ or *polA* mutant host producing π protein (Table 3). Thus, their properties are fundamentally different from those of plasmids containing the P2-201 and P2-203 mutations. This result led us to determine next if P2-245-6-7 and P2-246 reduce the activity of the P2 promoter. By using the primer extension assay, we found that both mutations also reduce the strength of the P2 promoter four- to fivefold and that the presence of wt π does not change the activity of the mutant P2 promoter (data not shown). This result strongly suggests that P2 promoter activity is dispensable for replication and that mutations in the −35 region (P2-201 and P2-203) exert their effect by a mechanism other than alteration of the activity of this promoter.

P2-201 mutation does not affect π binding to the iterons. The P2-201 and P2-203 mutations might eliminate replication of a γ origin core plasmid by interfering with π protein binding, since their position overlaps the sixth iteron to which the π protein binds (13, 15, 20, 36, 37, 50). We directly examined this possibility by comparing the patterns of DNase I digestion of a wt γ origin and a γ origin carrying the P2-201 mutation over a wide range of π concentrations (Fig. 4). Both wt and mutated origins showed the same pattern of enhanced DNase I cleavage and regions of protection including the area of the mutation. The amount of π required for initial binding was the same for templates containing a wt or mutant P2-201 γ origin. Therefore, we concluded that, while possible, it is unlikely that the effect of the P2 promoter-down mutations on γ origin replication is due to weakened binding of π to the sixth iteron (see Discussion).

Copy-up π variants permit replication of a γ origin core containing P2 promoter-down mutations. From the results presented above, we concluded that the effect of P2-201 and P2-203 on replication is not due to decreased activity of the P2 promoter or weakened binding of π to the origin. We had previously shown that certain mutations and conditions that inactivate the γ origin can be suppressed when copy-up mutant forms of π protein are supplied instead of wt π (6, 35, 52, 53; see the Discussion and Table 2). Therefore, we next investigated whether the copy-up π mutations could activate the γ origin core carrying either the P2-201 or the P2-203 mutation. Transformation tests were performed with hybrid plasmids containing either the wt γ origin core (pMF32), mutation P2-203 (pMU3), or mutation P2-201 (pMU4). We first established helper plasmids producing different types of π protein, wt π or copy-up protein π113 or π200, in *polA* mutant and *polA*$^+$ strains. Moreover, helper plasmids were used which produce

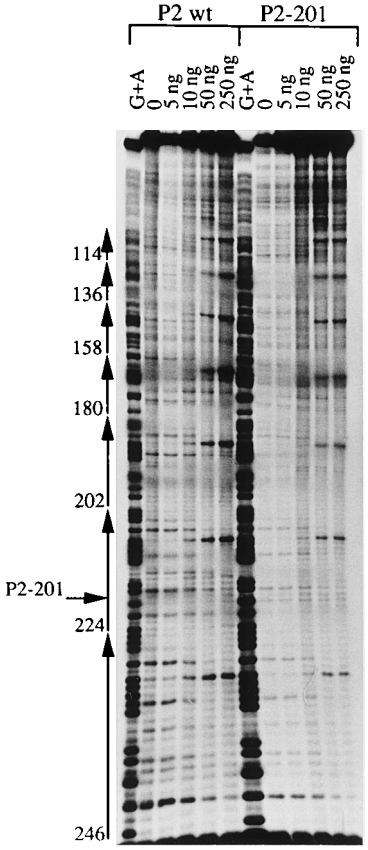

FIG. 4. Interaction of π with wt and mutant γ origin cores as determined by DNase I footprinting. Plasmid pMF32 (P2 wt) or pMU4 (P2-201) was linearized with *Eco*RI (the *Eco*RI recognition site is in the vector sequence directly downstream from position +277 of the γ origin core insert), labeled with ^{32}P at the 3′ end, and cut with *Hin*dIII (position +1 in the γ origin core). The amounts of π protein used in the reactions are shown above the lanes. A 40-ng sample of the indicated DNA was used in each reaction. The horizontal arrow indicates the position of the P2-201 mutation at coordinate +221. The vertical arrows show the positions of the seven 22-bp direct repeats. The coordinate number indicates the 3′ end of each repeat.

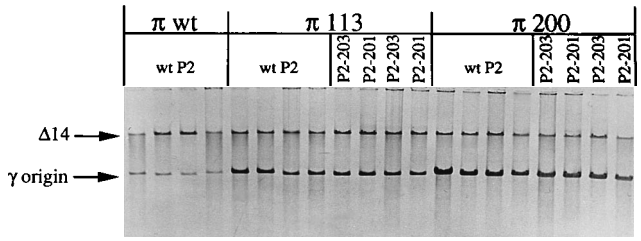

FIG. 5. Copy number analysis of γ origin core plasmids carrying the wt P2 promoter (lanes wt P2) or mutant P2-201 or P2-203 at Δ14 levels of wt π, π113, or π200. Arrows Δ14 and γ origin indicate helper plasmids producing low levels of π and the γ origin plasmids, respectively. Plasmids were linearized with *Sna*BI. At every level of π, there are four lanes with wt P2 which contain plasmids carrying the γ origin in two different orientations and in the presence or absence of the enhancer. Multiple lanes of P2-201 and P2-203 show plasmids carrying the mutant γ origin in two different orientations.

either low (Δ14) or high (Δ22) levels of wt or copy-up π. This approach allowed us to determine whether the effect of these three variants of π protein varies depending on their intracellular levels.

As shown in Table 4, all three γ origin plasmids transformed the *polA*$^+$ strain regardless of the level of π protein and the *pir* allele provided. These experiments tested cell competency and showed that none of the conditions employed permits runaway replication. Control transformations carried out with *polA* mutant recipients confirmed our earlier discovery that the Δ22 level of wt π inhibits the wt γ origin core replicon (pMF32) (52) and that both mutations P2-201 and P2-203 render the γ origin core plasmids inactive even at the Δ14 levels of wt π (as in Table 2). In sharp contrast, all three γ origin plasmids (pMF32, pMU3, and pMU4) can be established in the presence of π113 or π200 supplied at a wide range of levels. We also determined plasmid copy number in the presence of copy-up π. As shown in Fig. 5, copy-up mutants cause a similar increase in copy number for plasmids carrying a wt (pMF32) or mutant (pMU3 and pMU4) γ origin core. Thus, copy-up π proteins not only rescue plasmids containing a mutated γ origin but completely restore their replication abilities. These data indicate that copy-up π proteins are able to activate the γ origin core carrying the P2-201 and P2-203 mutations at a high or low level of π.

wt π and copy-up π mutants bind similarly to the iterons in vivo. Copy-up π mutants might permit replication of the γ origin core plasmids containing P2-201 and P2-203 mutations if their in vivo interactions with iterons differed in any way from that of wt π. To examine this possibility, we compared the in vivo methylation protection patterns of the γ origin iteron cluster in the absence or presence of either wt π or several copy-up π proteins. This was achieved by transformation of strain LE392 (*polA*$^+$) with plasmid pMF33 (containing both the γ origin and the pUC origin). The copy number of this plasmid is not changed by wt or copy-up π, since this plasmid replicates via the pUC origin. Exponentially growing cells containing various plasmid combinations (Fig. 6) were subjected

TABLE 4. Transformation efficiency of plasmids with wt or mutant γ origin core into *polA*$^+$ and *polA* mutant strains containing wt or copy-up π

Transforming plasmid DNA	γ *ori* genotype	Transformation efficiency[a]						
		polA$^+$ W3110 (Δ14-*pir*wt)	*polA* mutant					
			p3478 (Δ14-*pir*wt)	p3478 (Δ14-*pir*113)	p3478 (Δ14-*pir*200)	p3478 (Δ22-*pir*wt)	p3478 (Δ22-*pir*113)	p3478 (Δ22-*pir*200)
pMF32	wt P2 core	3.4×10^3	2.2×10^3	1.1×10^4	1.0×10^4	0	1.3×10^4	8.9×10^4
pMU3	P2-203 core	3.6×10^3	0	5.5×10^3	6.4×10^3	0	2.3×10^3	3.8×10^4
pMU4	P2-201 core	3.1×10^3	0	1.3×10^4	9.4×10^3	0	1.8×10^3	2.9×10^4

[a] 10^8 competent cells were transformed with 200 ng of the indicated plasmid DNA and plated on Luria-Bertani medium plates containing tetracycline (15 μg/ml) and penicillin (250 μg/ml). Transformation efficiency is expressed as number of colonies per microgram of DNA per 10^7 cells plated in one experiment. Similar values were obtained in four other experiments.

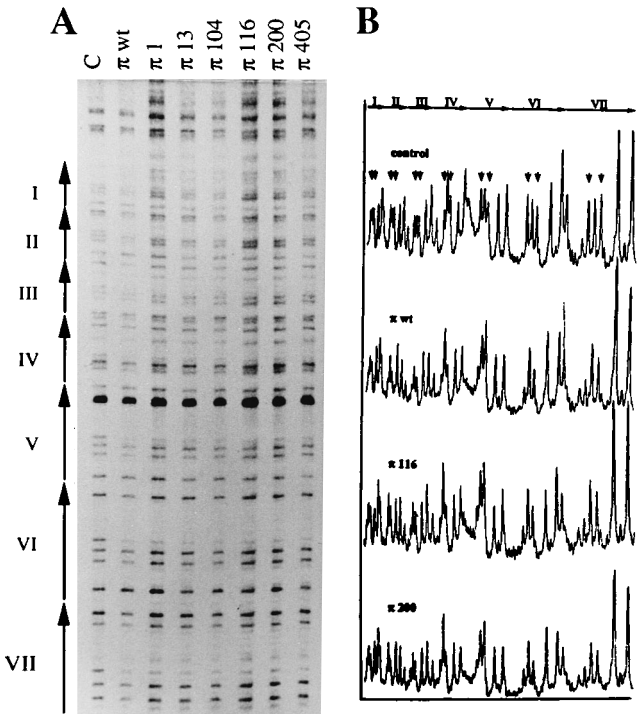

FIG. 6. In vivo binding of wt and copy-up π proteins as detected by DMS modification of DNA. Cells harboring different π-producing plasmids and the pMF33 plasmid were treated as described in Materials and Methods. (A) DMS pattern of the γ origin core region in the absence of π (lane C) or in the presence of wt π or different copy-up π proteins, as indicated above the lanes. The vertical arrows represent seven iterons. (B) Each lane in panel A was scanned with a densitometer, and profiles obtained in the absence or presence of wt π and two representative copy-up π proteins are shown. The vertical arrows indicate the positions of protected Gs; the horizontal arrows represent seven iterons.

to DMS treatment, and the resulting methylation patterns were analyzed by gel electrophoresis followed by densitometric scanning (see Materials and Methods).

The methylation protection patterns (Fig. 6) in samples obtained from cells producing or not producing π differed. The most prominent changes occurred at G-7 and G-9 of each 22-bp iteron unit. These positions are undermethylated in samples producing the π protein. Notably, the patterns of protection throughout the segment containing the iterons were similar for wt π and copy-up mutant π. However, the degree of undermethylation is not equal for G-7 and G-9 in all seven iterons. Rather, it occurs in a polar fashion, with the strongest undermethylation of G-7 and G-9 in iteron 2 and ever-decreasing affinity in iterons 3 to 7. G-7 and G-9 in iteron 1, which is adjacent to an A+T-rich origin segment, appears to be less strongly protected by the π protein. Since G-7 and G-9 were also protected in vitro by both a purified π–β-galactosidase fusion protein (19) and the native wt π protein (9), we conclude that undermethylation of G-7 and G-9 is also a consequence of π binding to these nucleotides in vivo. Similar methylation patterns observed with all of the π proteins examined indicate that copy-up mutations alter neither the binding selectivity nor the binding constant of the π protein.

DISCUSSION

The P2-201 and P2-203 mutations do not prevent replication of the γ origin core by reducing the strength of the P2 promoter. We show here that a four- to fivefold reduction in the strength of the P2 promoter results from the P2-201 and P2-203 mutations. These two promoter-down mutations inhibit replication of γ origin core plasmids. In contrast, mutations in the −10 hexamer (P2-245-6-7 and P2-246), which also reduce P2 promoter activity four- to fivefold, have no effect on replication. Hence, the P2 promoter is not required for γ origin core replication. Instead, it seems that the P2-201 and P2-203 mutations are pleiotropic point mutations that inactivate the γ origin by an unknown mechanism, in addition to decreasing the activity of the P2 promoter.

We considered the notion that the P2-201 and P2-203 mutations might interfere with π binding since they change the nucleotide composition of one of the seven π binding sites. We examined π protein binding to DNA templates containing these mutations by using a DNase I digestion assay (Fig. 4). We detected no altered π protein binding to the mutated iterons. In addition, several other findings make it unlikely that the observed replication deficiency resulted from weaker or altered π binding to the iterons in vivo. First, the P2-203 mutation alters a base pair that is not conserved among the seven iterons. Second, these mutations do not alter the two nucleotides most critical in π binding to the repeats (G-7 and G-9 of the 22-bp iteron [36; Fig. 6]). Third, a substantial reduction in the intracellular concentration of π would be expected to decrease π binding to iterons or change iteron occupancy. However, activation of the γ origin core is observed at a lower intracellular π concentration but not at a high level of π (52). Fourth, the function of a mutated γ origin core is not restored by an elevated level of π protein.

Other possibilities remain to be explored. (i) The P2-201 and P2-203 mutations might interfere with the formation of specific structures of the origin DNA required for its function. (ii) Binding of host-encoded factors might be altered. The latter idea seems compatible with the finding that the DnaA and IHF proteins bind to and are required for activity of the γ origin core (6, 32, 52). However, the consensus sequences for these two proteins are a considerable distance downstream of the mutated −35 segment of the P2 promoter. Moreover, the mutations in the −10 segment are much closer to the binding sites for the DnaA and IHF proteins but they have no detectable effect on replication.

Transcription originating in the γ origin. Another important aspect of our work is the demonstration that the P2-driven transcript is the sole RNA made in the left-to-right orientation within the γ origin core. This observation addresses the controversy regarding the source of transcription within this origin. Other investigators have identified a set of related transcripts, called activator RNAs, which were proposed to start at nt +31 (41), approximately 100 nt upstream of the P2-driven transcript. We detected no such transcripts in gene fusion experiments (37). Instead, we offered the simple explanation that these RNA species resulted from inadvertent creation of a new promoter by joining the γ origin and pBR322 sequences (37). Here we present biochemical evidence consistent with the hypothesis that γ origin transcription originates exclusively from the P2 promoter. Other investigations have indicated that transcription, whether dependent on or independent of the P2 promoter, is not essential for replication of the γ origin since in vitro replication of a γ origin template is not inhibited by rifampin (30, 32). In view of these data, it remains to be determined what role, if any, the P2 promoter plays in γ origin replication and/or π protein production. It is consistent with the location of the P2 promoter to assume that P2, in addition to the P3 promoter (Fig. 1), could participate in expression of the *pir* gene (10, 54). Additional support for this scenario stems

from the fact that there is no transcriptional terminator between the P2 and P3 promoters (47).

Reassignment of the −35 hexamer of the P2 promoter. Since a single C-to-T nucleotide change (P2-203 mutation) confers the promoter-down phenotype, we initially assumed that this substitution alters the −35 hexamer consensus. Thus, coordinate +221 was identified as the leftmost nucleotide of the −35 hexamer of the P2 promoter (37; Fig. 1). We have now mapped nt +1 of the P2-driven transcript, and this identification seems incorrect for several reasons. First, the previously assigned −35 hexamer is a poor match to the consensus for *E. coli* promoters. Second, the spacing between −10 and −35 hexamers is 18 bp, instead of the optimal 17 bp. Third, if this were the −35 hexamer, promoter-down mutations P2-201 and P2-203 would increase instead of decrease the number of matches to the consensus, thus increasing promoter strength instead of decreasing it. Taking all of these factors into consideration, we propose that the −35 hexamer of the P2 promoter starts at the G at position +222 (Fig. 1). This newly assigned sequence has a reasonable match to the consensus −35 hexamer (GTGAAA [four matches to the consensus underlined]) and also improves the spacing between the −10 and −35 hexamers to 17 bp (Fig. 1). Moreover, the P2-203 mutation decreases the number of matches to the −35 consensus. However, the P2-201 mutation now lies outside of the consensus (position −37). This is a highly unusual position for a mutation causing a promoter-down phenotype. The properties of this mutant indicate that the P2 promoter might be positively regulated by a factor that binds in its vicinity or that the P2 promoter might need an additional sequence upstream of the −35 region that acts in a factor-independent manner.

There is precedence for alterations at position −37 which affect promoter strength. Promoter-down mutations in the *lac* promoter (29) and in the *rrnB* P1 promoter (25) have been identified which, like P2-203, contain substitutions at position −37. The base substitution at position −37 affects the basic strength of the *rrnB* P1 promoter (25). Similarly, in the *lac* promoter, mutation affects the basic promoter strength, since it also affects the *lacUV5* promoter, whose activity is CAP independent (41a). In the case of the P2 promoter, however, activation by a putative factor seems likely, since this promoter is silent in vitro (data not shown) but active in vivo.

Copy-up mutant forms of π allow replication of plasmids carrying P2-201 and P2-203 mutations. The P2-201 and P2-203 mutations have to be considered the only known point mutations of the γ origin which totally inactivate its function in the presence of wt π. Other mutations rendering the γ origin nonfunctional contain either multiple changes or deletions or insertions of various lengths. As we show here, the replication deficiency resulting from these unique mutations is totally reversed when wt π is replaced by copy-up π variants.

How might this effect be achieved? One possibility is that copy-up π mutants differ from wt π in the way they interact with the iterons. Although in vitro binding analysis is inconsistent with such a possibility (14, 30a), the in vivo situation may be different. Binding might be affected by many factors, including the presence of other proteins, nonspecific DNA binding, association of subunits, and ligand-binding equilibria. Thus, we tested this possibility with a methylation protection assay in vivo and found that the pattern of protection is similar for wt π and copy-up mutant π. A somewhat unexpected outcome of these experiments is the finding that the binding of π to iterons occurs in an ordered fashion; the second iteron is bound first, and then the third through the seventh are bound. π binds to the first iteron last. This finding appears to be different from the in vitro analysis of π binding to the iterons. In vitro, no single iteron showed higher affinity for π than the other six (15). An ordered fashion of π binding in vivo could be due to the presence of other plasmid or host factors and might be important for control of replication. The order is the same for wt π and copy-up mutant π. Furthermore, copy-up mutations do not seem to change any aspect of π binding to iterons in vivo, which would have been detected by the DMS methylation assay. Hence, the mechanism which might be responsible for reversing the effect of P2-201 and P2-203 mutations on replication of the γ origin core remains unknown. Several observations, summarized in Table 5, indicate that the requirements for γ origin function are considerably less stringent in the presence of copy-up mutant π. Thus, as we have pointed out earlier (6, 11), copy-up mutant π may utilize an alternative pathway of initiation of DNA replication which is not utilized by wt π or is utilized only under certain circumstances.

TABLE 5. Ability of γ origin core plasmids containing different mutations to replicate in the presence of wt or copy-up π proteins at low and high protein levels

Genotype	Replication[a]				Reference or source
	wt π		Copy-up π		
	Δ14	Δ22	Δ14	Δ22	
wt γ origin core	+	−	+	+	52
P2 promoter-down mutations	−	−	+	+	This work
IHF site 1 mutation	−	−	+	−	6a
IHF-deficient host strain	−	−	−	−	6a
wt γ origin core + enhancer	+	+	+	+	14
DnaA box 1 mutation	+	−	+	+	53
IHF site 1 mutation	+	−	+	+	6a
IHF-deficient host	+	−	+	+	6
Extra set of repeats in *cis*	+	−	+	+	35
Iteron mutations weakening π binding	NT	−	+	+	34
Iteron mutations eliminating π binding	−	−	+	+	34
Alleviation of incompatibility	−	−	+	+	35

[a] +, plasmid can replicate; −, plasmid cannot replicate; NT, not tested.

Interestingly, we found that not only copy-up mutant π but also the enhancer sequence reverses the effect of P2-201 and P2-203 mutations. It is tantalizing to speculate that the enhancer may confer yet another mode of initiation of DNA replication at the γ origin. Further biochemical analysis of the replication mode dependent on wt π and its copy-up mutant forms is necessary to elucidate the mechanism(s) used by π to control the initiation of DNA replication.

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